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13. ABSTRACT (Maximum 200 words)

We report on the research funded in part by this ARO grant towards developing an all-optical terahertz biosensor. This program is motivated by the need for fast and adaptable biosensors that do not require probe labeling. Our sensors are based on an intrinsic change in the probe molecule's physical characteristics upon target binding, namely the THz absorption. The proposed sensor consists of a compact THz spectroscopy system based on electro-optic imaging of a sample matrix . The sample matrix made from xerogel would have defined regions of probe molecules as well as regions for referencing. This sample substrate would allow for both high THz optical density and easy access of airbourne targets to bind to the embedded probe molecules. The stated goals in the original proposal for this first funding period were as follows: (1) spectral benchmarking of probes: free and complexed focusing on benign targets to establish sample protocols, (2) initial xerogel array development and(3) initial construction of EO imaging system. All three of these initial goals have been achieved. We will discuss spectral benchmarking for lysozyme/N-acetylglucosamine, anti-lysozyme/lysozyme, deoxy cytochrome c/oxygen and deoxy myoglobin/oxygen binding. We will also present our xerogel characterization as a function of hydration. In all cases we see a significant change in the THz absorbance with ligand binding, supporting the suggested stragegy of using THz absorbance as a labeless method of biodetection. In the coming year we will attempt to print a xerogel with different proteins and then expose the gel to aerosols of target molecules to determine binding efficiency for this sample prep. We will attempt to detect the binding both with standard THz spectroscopy as well as using THz imaging. The highest impact results include establishing that the THz dielectric response highly resembles the distribution of normal modes for proteins and can be modeled using a continuous distribution of oscillators with nearly uniform oscillator strength. We have demonstrated that the THz dielectric response is sensitive to conformational intermediate states for photoactive proteins, to ligand binding for lysozyme and to oxidation state for cytochrome c.

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(C) FINAL PROGRESS REPORT

(4) Statement of Problem Studied

We have used the ARO grant #DAAD19-02-1-0271 to explore the development of biosensors based on terahertz dielectric response.[1-11] These sensors exploit the fact that upon binding between a probe molecule and target molecule there will be a change in the density of vibrational modes associated with large-scale structure. Biosensing systems use chemically specificity of binding between the target and the probe as a method of determining the target's presence.[12] Sensing of the binding is through either an innate change of the probe's optical or electrical properties, or through the tagging of the probe or target with markers, most often fluorescent markers. Innate changes in the probe response vary for different probe-target interactions, thus the biosensing method is often dependent on the pathogen under consideration and any given platform is not able to address all possible pathogens.

Universally applicable biosensing systems are only now being considered and developed, the most successful and well known to date is the microcantilever system.[13] Here, one side of the microcantilever is functionalized with the probe molecule. The change in mass with target binding is sensed by the deflection of the cantilever. Thus by using an innate physical characteristic that must change for any probe-target binding, the microcantilever system is nearly universally applicable and does not require tagging of the probe or target molecules. However the microcantilever system has requirements of vibration isolation and cantilever manufacture and functionalization by the probe molecules. Further, the probe molecules must be bound to the cantilever surface. This requires development of the proper chemistry to achieve the oriented binding so the interaction surface is still available for target binding.

Another property of the biomolecular system that must necessarily change with probe-target binding is the low frequency vibrational modes associated with large-scale motion of the system. That is vibrational modes in which entire structural subunits beat against one another. The frequencies for the structural or conformational modes lie in the far infrared or terahertz frequency range (2 - 200 cm^{-1}, .06 – 6 THz).[14] With probe-target binding, one would expect that the new bound system will differ from the free probe in mass, net dipole moment, and conformational normal mode spectrum. We note that the local vibrational modes lying in the MIR and the electronic excitations in the UV/Visable regions may only be slightly effected by bindng since the interactions giving rise to these excitations are at a local level, whereas the conformational vibrational mode response probes the overall molecular system. Key in this development is the realization of a table top terahertz dielectric characterization systems, terahertz time domain spectroscopy (TTDS).

With the support of ARO funding we have demonstrated that low-frequency vibrational modes of proteins are optically active in the THz frequency range.[9-11, 15] While we do not see narrow features associated with specific conformational modes, we did observe that the THz absorbance strongly resembled the calculated normal mode spectrum. This strong resemblance suggests that all modes are optically active and that the absorbance is a measure of the normal mode spectrum and thus sensitive to ligand binding. This report is an overview of the accomplishments under ARO funding: establishment of the necessary sample preparation protocols and environmental controls to achieve reproducible results; determination of hydration effects on terahertz response; determination of terahertz sensitivity to protein conformation,

ligand binding and oxidation state; and the investigation of xerogel substrates as platforms of terahertz biosensing systems.

(5) Summary of Most Important Results

Preparation Protocols

A key concern with respect to both sensor technology and terahertz spectroscopy is biomolecular sample preparation. For biosensor applications sample collection should be simple. For terahertz the main concerns are water content. As the absorption coefficient for liquid water is 200 cm^{-1} at 1 THz, it is essential to minimize water. Dried samples are preferred, but the question then arises whether dried proteins will behave the same as hydrated proteins and most imporatntly will the flexibility changes that one might expect for hydrated proteins upon ligand binding still occur, or will they be reduced and/or eliminated with dehydration. Further samples must avoid optical artifacts such as scattering enhancement and thickness nonuniformity. We have considered four types of sample preparation: pressed lyophilized powders, high concentration solutions, hydrated films and proteins embedded in xerogel substrates.

Pellets of lyophilized material while the most straight-forward preparation are not desirable for both practical and scientific reasons. Firstly lyophilized materials are structural different than hydrated proteins and careful structural measurements as a function of rehydration have not been performed to determine at what point native conformation is achieved. It is difficult to systematically measure a single pressed pellet as a function of hydration or ligand binding and in general additional pellets must be made for each set of conditions. Finally monitoring of the system by secondary UV/Vis probes is not possible with pressed pellets.

It has been difficult to date to distinguish the protein contribution above the solvent background for high concentration solutions. We have recently undertaken systematic measurements on 200 mg/ml lysozyme solutions with varying water:glycerol concentrations. We do see a transition in the apparent displacement volume of the lysozyme with increasing glycerol content. However this contribution appears to be frequency independent.

Hydrated films of proteins have given the highest quality terahertz response data. It was determined that not all proteins form films with the same quality and sample uniformity is the chief issue for these samples. The films allow for secondary probes of the samples to establish native state population, ligand binding, and oxidation state. The hydration of the films is readily controlled and systematic measurements of hydration have some resemblance to results of Pethig and coworkers working in the MHz range.[16]

Key issues to overcome with the films have been unformity without cracking. It was found that standard drying often resulted in “the coffee drop effect” where the film is thick at the outer perimeter and then very thin inside this perimeter. In addition we found that in some cases films formed peaks at the center. These effects were overcome by slowly dehydrating the films in a dry nitrogen atmosphere or in a refridgerator with parafilms covering the pipetted drop with pin-holes directly above the center of the drying drops. In addition, adding a small percentage of glycerol (~1-2 %) aided in the uniformity of the films.

Xerogels have been made an intially characterized. High concentrations were achieved with both free protein and protein bound with a ligand. This method is the most applicable to biosensors.

Hydration Control

It was found that the terahertz response was highly dependent on hydration level.[2, 10, 15] This dependence arises from several sources. Increase of intermolecular coupling mediated by the bound waters. Individual water molecular dielectric relaxation response contribution, and bulk water contribution. The frequency response difference between bulk water and hydrated protein suggests that the bulk water does not strongly contribute to the dielectric response. Further the hydration levels used for these studies was not sufficient for bulk water type properties as has been demonstrated by previous investigators who found no discontinuity in specific heat measurements at 273 K for proteins hydrated to the same levels.

This large dependence on hydration for collective mode response requires critically controlled measurements. A fact often neglected in the literature. We have found that saturated salt solutions are unreliable and that high flow rates are difficult to establish with these systems. Using funds provided by the ARO grant we purchased a Licor Dew Point generator. This single piece of equipment has already resulted in the preparation of two manuscripts for publication. We are able to continuously control the relative humidity in the sample chamber from 0 to 100 %.

The ability to control the humidity is not sufficient to ensure the reliable hydration level of the protein films and xerogels. It is imperative to know the equilibriation time for hydration. Using thermogravimetric analysis instrumentation available to us, we have found for protein films this equilbriation time is 1 h for dehydration and 2 h for hydration.

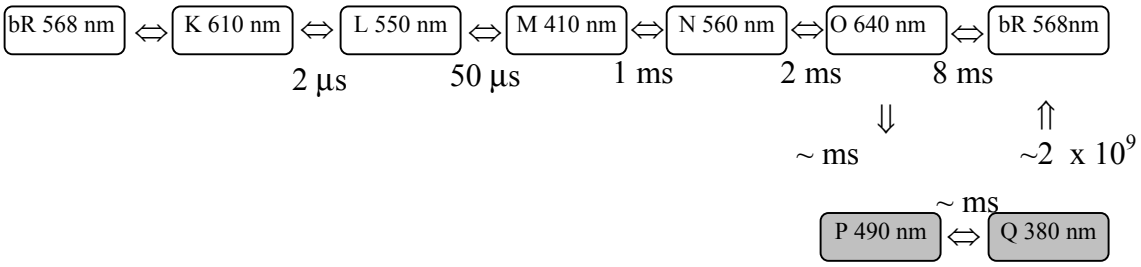

Figure 1. Photocycles of wild type bacteriorhodopsin at 292K and pH 7.

Conformational State Sensitivity

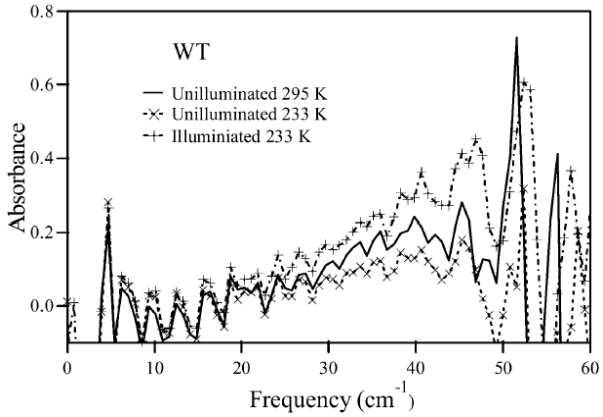

Figure 2. Temperature and conformation dependence of a thin film of WT bacteriorhodopsin

A first step towards the demonstration of the dependence of THz dielectric response on protein low frequency mode density is the dependence on protein conformation. Clearly if the overall structure of the protein changes, one might expect a change in the protein dynamics. Further, comparing the terahertz response for a protein film as a function of conformation removes artifacts due to sample to sample variation. For these measurements we used bacteriorhodopsin (BR). BR is a photoactive membrane protein from the archaebacteria *Halobacterium salinarum*.[17] The photocycle of BR is shown in Figure 1.

The main photocycle is denoted by the resting state bR and then a series of photointermediate states K, L, M, N, O and returning to the resting state bR. The main photocycle serves to pump protons from the intracellular to extracellular side of the membrane as a method of energy storage for anaerobic metabolism. In the figure the intermediate state is denoted its UV/Vis absorption maximum and lifetime for pH 7.0. As seen in the figure a branched photocycle also exists with state P and Q.

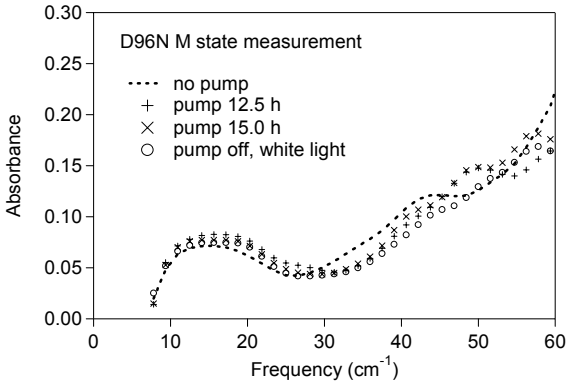

Figure 3. Terahertz absorbance measured as a function of M state population for D96N film at 100% r.h., room temperature.

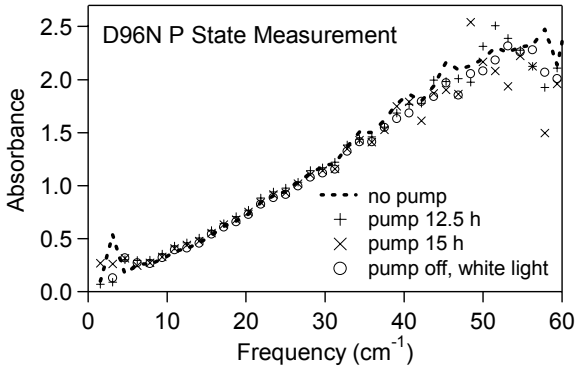

Figure 4. Terahertz absorbance measured as a function of P state population for D96N film at 100 % r.h. and room temperature.

We performed a series of TTDS measurements on bacteriorhodopsin and D96N films to determine if the FIR absorbance is affected by conformational change of a protein, if two mutants of a protein can be distinguished and if the FIR absorbance is related to function.[10] The measurements were performed in the steady state by cooling the BR and D96N films to -40 ºC and photoexciting the samples. At -40ºC the M state of WT BR is stable. In Figure 2 is shown the substantial increase in the THz absorbance for the WT in the M state observed, but no change was observed in the absorbance for D96N, see Figures 3 and 4. It was speculated that the increase in absorbance was related to an increase in the low frequency normal mode density and therefore the flexibility of the protein. The lack of such increase for the D96N could suggest that the M state is not as flexible for the mutant inhibiting its ability to access the necessary conformations for completion of the photo-cycle. Such a difference in intermediate state flexibility would be in agreement with a slower photo-cycling time for D96N. These measurements establish that the previously observed difference in the FIR response is fundamental to the mutation and that the dynamics of the two systems is fundamentally different.

Ligand Binding Sensitivity

Critical to realizing a biosensor based on THz spectroscopy is the demonstration of sensitivity to ligand binding. Measurements of the THz dielectric response for hen egg-white lysozyme (HEWL) as a function of bindng with tri-N-acetyl-D-glucosamine (TriNAG) have been made.[5, 6] We measured the response as a function of hydration and binding at less than 5% relative humidity (r.h.) and 63% r.h.. We observed the absorbance decreases with binding and the index is nearly independent with binding. We then compare these results with a phenomenological theory to quantify the observed dielectric response.

In Figure 5 we show the index measurements for representative films. As seen there is very little contrast between the unbound and bound HEWL films. The strong increase in the index with hydration is similar to what we have seen earlier with other proteins. The hydration is

not sufficient to have free water in the sample, thus the change in index is due the fundamental change of the dielectric response of the protein with bound water. We will discuss the possible source of this increase in the modeling section.

In Figure 6 we see a strong contrast between the absorption coefficient for the unbound versus bound films. There is a net decrease in the absorption coefficient with binding. We see that this contrast continues for the hydrated films. As has been seen in previous studies the hydrated protein has a higher absorbance. The decrease in absorption with binding could come from a blue shifting of the normal mode density with binding, or a decrease in dipole coupling. We consider these two affects in the modeling section.

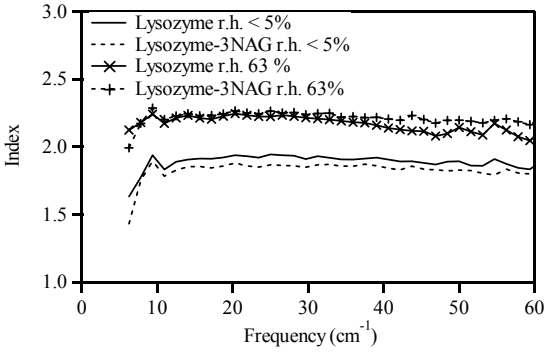

Figure 5. THz index measurements for HEWL and HEWL-TriNAG films for dry and 63% relative humidity.

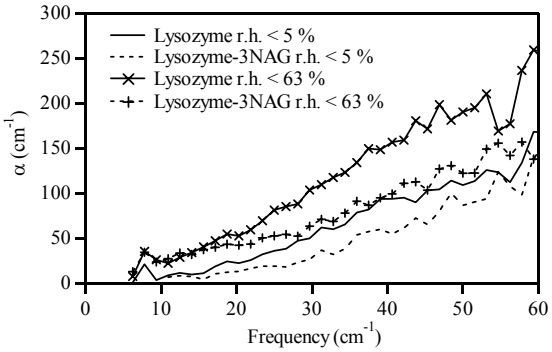

Figure 6. THz absorption coefficient for HEWL and HEWL-TriNAG films determined using Eq. 2.1.

In understanding the results we begin by calculating the normal mode spectrum using molecular mechanics. Starting from the X-ray coordinates (1BWH.pdb), the structure is minimized and we calculate the normal modes using CHARMm 27b3. The histogram of the calculated normal modes is plotted with the measured absorbance for a lysozyme film in Figure 7. As seen in the figure, while the two do not precisely overlay the overall trends are similar, that is a broad distribution increasing with frequency. This result suggests that the oscillator strength does not strongly depend on the low frequency mode in this range and that the absorbance spectrum directly measures the normal mode density. In general, the dielectric response for a collection of oscillators is given by:

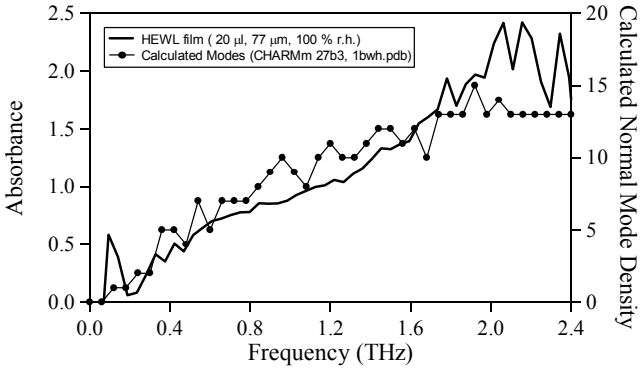

Figure 7. Comparison of the normal mode density and the absorbance measured for one of the HEWL films.

$$\varepsilon(\omega) = \varepsilon_0 + \frac{Nq^2}{m}\sum_j \frac{\mathrm{f}_j}{(\omega_j{}^2 - \omega^2) - i\gamma_j\omega} \qquad 1.1$$

f_j is the dipole matrix element for the transition at ω_j. Combining the charge q, mass m and number of oscillators N with f_j we can define a net oscillator strength, f_j. For a macromolecule such as a protein, there are many overlapping conformational modes in the low frequency region

giving rise to the monotonic increase in normal mode density seen in Figure 5. In order to model the response we assume that the normal mode density linear increasing function with frequency with slope n and that the oscillator strength and damping are frequency independent, f and γ. Thus dielectric response can be expressed as an integral with n(ω) as the density of normal modes in the frequency interval of dω:

$$\varepsilon(\omega) = \varepsilon_0 + \int_0^\infty \frac{nfd\omega'}{(\omega'^2 - \omega^2) - i\gamma\omega} = \varepsilon_0 + \int_0^\infty \frac{\mathrm{f}d\omega'}{(\omega'^2 - \omega^2) - i\gamma(\omega')\omega} \qquad 1.2$$

where we note that increasing the slope of the normal mode density or increasing the oscillator strength have an identical effect on the dielectric response so that we replace the product *nf* by a single parameter f. We now consider how the index and absorption coefficient change as we vary f and γ determined using:

$$n = \mathrm{Re}\sqrt{\varepsilon(\omega)} \qquad \alpha = 2k_o \mathrm{Im}\sqrt{\varepsilon(\omega)} \qquad 1.3$$

In Figure 8a) we show the dependence of the index on these parameters. In the large damping regime the index is increasing at low frequencies and flattens out and decreases with increasing frequency similar to that seen experimentally. One might expect to be expected in a high damping regime given the high intramolecular and intermolecular coupling expected for these dense samples. The absorption coefficient calculation in Figure 8b) suggests that a net decrease

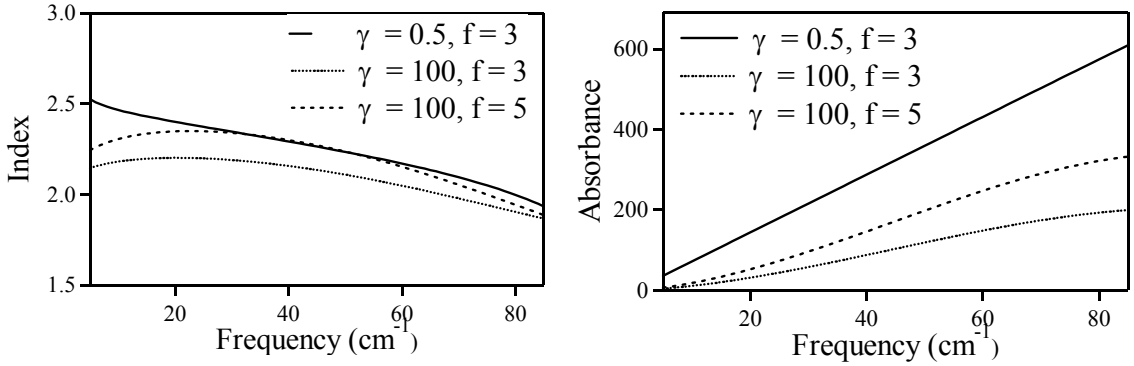

Figure 8. Calculated a) index and b) absorbance using Eqs. 1.2 and 1.3.

in the parameter f agrees with the observed decrease in absorption.

The change in dielectric response we observe for HEWL and HEWL-TriNAG films may originate from a) change in normal mode density at low frequencies à decrease in flexibility, b) decrease in oscillator strength and/or c) increase in damping with binding. Comparing the data with a simple model we see that change of dielectric response is most consistent with a decrease in normal mode density at low frequencies (a decrease in flexibility) or a decrease in the net dipole moment with binding. The net change in dipole can be determined by lower frequency dielectric measurements. These results demonstrate that dielectric response may be used as an indicator for binding.

Oxidation State Sensitivity

In order to verify if THz spectroscopy is sensitive to flexibility changes observed by other techniques, we examined the terahertz dielectric response dependence on oxidation state for cytochrome. X-ray (PDB files 3CYT and 5CYT), NMR (PDB files 1OCD and 2FRC) and compressibility measurements have demonstrated that the flexibility of cytochrome c increases with oxidation.[18, 19] We find that the terahertz response is highly sensitive to the oxidation state of cytochrome c and the increase in absorbance and index is consistent with and increase in flexibility. We demonstrated that this change can not arise from a difference in the equilibrium water content for the two oxidation states. This is the strongest proof to date that terahertz dielectric response is sensitive to protein flexibility.[3, 7]

We performed measurements on films of oxy and reduced cytochrome c and observe a decrease in both the THz absorbance and index in the reduced system. Furthermore as the reduced film oxidizes in time, the dielectric response moves towards the oxy sample, demonstrating the change is not due to the reducing agent, still present in the reduced film. Typical absorbance and index measurements are shown in Figures 9 and 10 respectively. The absorbance is normalized the thickness of the film. As seen there is a sharp contrast between the oxidized and reduced samples. The decrease in absorbance could come from either a strong decrease in dipole moment, decrease in the low frequency density of modes or both. Such a decrease in normal mode density is in agreement with the decrease in flexibility measured by X-ray B-factors. The index strongly increases with oxidation, from ~1.5 to ~2.0 at 1 THz.

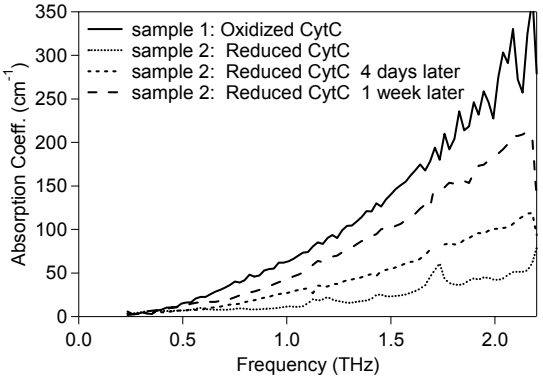

Figure 9. Absoption coefficient of cyotchrome c oxy and reduced thick films. Measurements performed at 298K and < 5% r.h.

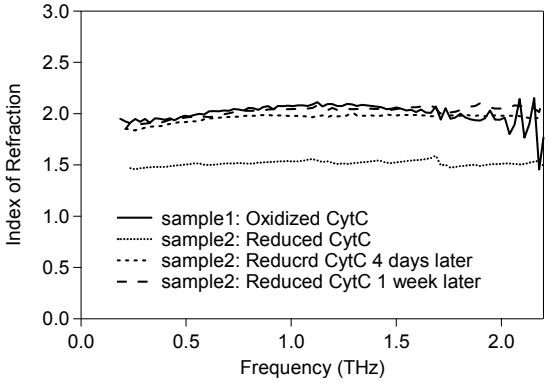

Figure 10. THz frequency index for cytochrome c films determined from TTDS measurements at 298 K and < 5% r.h.

Xerogel Substrates

We have demonstrated that THz absorbance and dielectric response is dependent on ligand binding for thin films.[1] In these studies, solutions of probe molecules and probe molecules complexed with a target were used to form thin films. In real world applications this type of sample preparation would be impractical. Instead one would wish to monitor the THz transmission

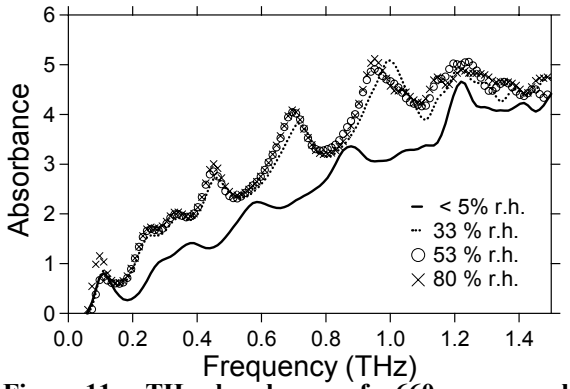

Figure 11. THz absorbance of a 660 μm xerogel plate as a function of relative humidity.

of immobilized probe molecules on a substrate and the presence of the target bound to the probe would be determined by the change in the THz transmission. Key to the realization of any biosensor system is the access of the target molecules to the sensor. Thin films of probe molecules would provide limited target access in that the binding would be mainly confined to the surface of the film. It would be preferable to have a substrate in which both the probe is encapsulated, and the target has ready access to the probe. We investigated the application of probe molecules embedded in xerogels as a method to increase target access and optical density. Xerogels are high porosity nanoporous silica films. Biomolecular inclusion in xerogels has been demonstrated and in most cases the biomolecule behaves as in a dilute solution.[20] The pore size for these xerogels is determined by the embedded biomolecule and the silica network allows for sufficient access to the embedded biomolecule so that reactions such as change of pH occur as if in solution. The use of xerogels not only eliminates the concern for target access it also allows thick samples to rapidly reach their equilibrium hydration at a given atmospheric relative humidity. Xerogels have not before been characterized in the THz frequency range. A key question is how the transparency is affected with relative humidity. For example, fused quartz windows are nearly opaque in the THz range due to water within the glass. An additional question is the absorbance of proteins measured in the xerogels versus that measured either in thin films or pellets. Here we report our measurements of the absorbance and index measured for xerogels as a function of relative humidity and how the transmission of the xerogels are affected by biomolecular inclusion, using hen egg white lysozyme (HEWL) as our model biomolecule. The data shows that the xerogels are sufficiently transparent in the THz range for use as substrates. The biomolecular inclusion does not affect the transmission of the xerogel in a simple additive way, but rather changes the nature of the dielectric response of the system as a whole. We suggest that this change is due to a change in the way water can bind to the xerogel in the presence of the biomolecule.

In Figure 11 we show the absorbance derived from the measured transmission for the 660 μm thick xerogel for relative humidities of < 5% to 80 %. The large absorbance increases with frequency similar to the dielectric relaxation type response seen for many glasses. The periodic peaks seen in the data are due to multiple reflection (etalon) effects in the xerogel plate. This type of absorbance may arise from localized collective vibrations within the glass or from water that is still trapped within the glass.

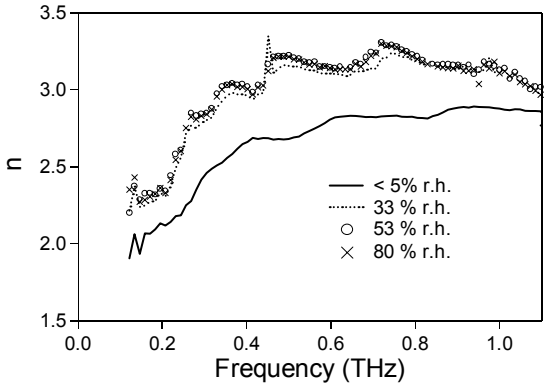

Figure 12. Index measured for 660 μm thick

As the humidity increases it is expected that the absorbed water will both contribute to an increase in absorbance and index. As seen in Fig. 11 the absorbance does increase somewhat for 33% r.h. but at higher humidities there is no further increase. This result is somewhat surprising and suggests that the additional water absorbed does not contribute to the THz absorbance. While the absorbance is high, the transparency of the xerogel is sufficient for THz measurements of embedded proteins.

In Figure 12 we show the index of the neat xerogel for different humidities. The dielectric constant increases with hydration and this increase has saturated by 33 % r.h. in agreement with the absorbance measurements. The shift in the dielectric constant is in good agreement with the shift in etalon fringes seen in the absorbance data. The net increase in

dielectric constant with hydration corresponds to an effective dielectric constant determined by the combination of the adsorbed water and the silica glass.

We have performed the first dielectric characterization of xerogels in the terahertz frequency range. The xerogels have an absorption coefficient of 174 cm^{-1} and index ~ 3.16 at 33 % r.h. and 1 THz which is sufficiently low to use xerogels as substrates for THz measurements of biomolecular samples. Inclusion of biomolecules into the xerogel does not result in a simple additive response, but rather the biomolecules within the xerogel define a new composite material with a distinct THz dielectric response. The relative transparency of the composite material suggests that xerogels are viable substrates for realizing THz biosensor systems.

Through extending a single year funding we were able to accomplish a great deal towards realizing a biosensor based on THz spectroscopy. Currently we have NSF funding to continue some of the more basic research aspects of this work and hope that we might attain further funding to support the realization of the biosensor.

REPORT DOCUMENTATION PAGE (SF298)
(Continuation Sheet)

(6) Papers submitted or published

(a) submitted, not yet published

1. "Protein Dynamics: Oxidation Effects on Terahertz Dielectric Response of Cytochrome C," J.-Y. Chen, J. R. Knab, J. Cerne and A. G. Markelz, submitted Nature 2004.

(b) published in peer reviewed journals

1. "Terahertz measurements of the Photoactive Protein Bacteriorhodopsin mutant D96N: M and P States," J.-Y. Chen, J. R. Knab, J. Cerne, J. R. Hillebrecht, R. R. Birge and A. G. Markelz, Journal of the Materials Research Society 2004.

2. "Protein Flexibility and Conformational State: A comparison of collective vibrational modes of WT and D96N bacteriorhodopsin," S. E. Whitmire, D. Wolpert, A. G. Markelz, J. R. Hillebrecht, J. Galan, and R. R. Birge, Biophys. J. **85** (2003).

3. "Terahertz Time Domain Spectroscopy of Biomolecular Conformational Modes," S.Whitmire, A.G. Markelz, J. R. Hillebrecht, and R. Birge, Phys. Med. Biol. **21**, 3797, 2002.

(c) conference proceeding

1. "Tagless and Universal Biosensor for Point Detection of Pathogens," Proceedings of the SPIE Security and Defense Symposium, April 12-16, 2004, Orlando, FL.

2. "Measuring Protein Flexibility with Terahertz Spectroscopy: Basic Research and Applications," Proceedings of the IEEE LEOS Summer Topical Meeting, June 28-30, 2004, San Diego, CA.

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(8) Report of Inventions

Not applicable

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